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Pyridoxylamine reactivity kinetics as an amine based nucleophile for screening electrophilic dermal sensitizers

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Abstract

Chemical allergens bind directly, or after metabolic or abiotic activation, to endogenous proteins to become allergenic. Assessment of this initial binding has been suggested as a target for development of assays to screen chemicals for their allergenic potential. Recently we reported a nitrobenzenethiol (NBT) based method for screening thiol reactive skin sensitizers, however, amine selective sensitizers are not detected by this assay. In the present study we describe an amine (pyridoxylamine (PDA)) based kinetic assay to complement the NBT assay for identification of amine-selective and non-selective skin sensitizers. UV-Vis spectrophotometry and fluorescence were used to measure PDA reactivity for 57 chemicals including anhydrides, aldehydes, and quinones where reaction rates ranged from 116 to 6.2×10^{-6} M^{-1} s^{-1} for extreme to weak sensitizers, respectively. No reactivity towards PDA was observed with the thiol-selective sensitizers, non-sensitizers and prohaptens. The PDA rate constants correlated significantly with their respective murine local lymph node assay (LLNA) threshold EC3 values ($R^2 = 0.76$). The use of PDA serves as a simple, inexpensive amine based method that shows promise as a preliminary screening tool for electrophilic, amine-selective skin sensitizers.

Keywords

Skin sensitization; Reactivity assay; Pyridoxylamine; Local lymph node assay

1. Introduction

Allergic contact dermatitis (ACD) is caused by a wide range of chemicals after prolonged or repeated contact with the skin. In developed countries, 15–20% of the population has contact allergy to one or more chemicals in their environment (Nielsen et al., 2001). Contact allergies constitute 20–50% of occupational contact dermatitis cases and it is estimated that ACD accounts for 7% of all occupations related diseases (Andersen, 2003; Jost, 2003).The main causes of ACD in the USA are the members of the Rhus genus (poison ivy, poison

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oak, and poison sumac), paraphenylenediamine, nickel, rubber compounds and ethylenediamine hydrochloride (Jost, 2003). Chemical-induced allergy thus remains an on-going challenge and an important occupational and general public health issue. People continue to be exposed to new chemicals making the identification of allergenic chemicals a priority.

Developed in the 1990s, the murine local lymph node assay (LLNA) (Gerberick et al., 2007a) is the preferred *in vivo* assay used for skin sensitization hazard identification and characterization. Even though the LLNA is now accepted as a standalone *in vivo* assay for evaluating potential skin sensitizers, recent changes in the European Union will require non-animal based toxicity testing before the marketing of consumer products such as cosmetics (EU Directive, 2012). There is, therefore, a strong push to develop non-animal based assays to screen products for their skin sensitization potential. The basis of these reactivity-based methods is that a compound must be able, either as such or after metabolic or abiotic activation, to react covalently with skin proteins (haptenation) to form a neoantigen. Despite considerable investment in exploring different approaches to develop alternative methods for skin sensitizer identification and characterization, no validated alternative methods are available to date. Nevertheless, a number of emerging *in chemico, in vitro* and *in silico* assays (Gerberick et al., 2004, 2007b) are showing promise for use in the identification and characterization of dermal sensitizers. Further exploration of these assays is warranted in view of the potential for their ability to detect and possibly measure the potency of skin sensitizers. Notably, several peptide reactivity based assays have been reported (Gerberick et al., 2004, 2007b) where the target moieties on the various peptides have usually been either cysteines or lysines. Model peptides have been used as surrogates for protein binding. Aptula et al. (2006) reported the use of glutathione as a model nucleophile to study the reactivity of several skin sensitizers. The direct peptide reactivity assay (DPRA) which measures loss of parent, unbound peptide after addition of an electrophilic chemical, has been nominated to the European Centre for the Validation of Alternative Methods (ECVAM) for validation after demonstrating good sensitivity and specificity (Aeby et al., 2010; Bauch et al., 2011).

A number of limitations associated with peptide reactivity based assays have been identified as discussed by Natsch et al. (2007). These include solubility incompatibilities between peptides and test chemicals, inability to directly monitor the chemical reaction kinetics in solution resulting in estimated rate constants and the non-specific modifications of the peptides due to oxidative reactions. Occurrence of false positives has been noted with peptide reactivity assays due to oxidative chemistry which may not be relevant to skin sensitization. Utility of HPLC-MS techniques (Aleksic et al., 2009; Natsch and Gfeller, 2008) can add specificity and eliminate false positives due to oxidation, but these add complexity to the assays while making them more costly. A recent review also discusses some of the limitations of these assays (Roberts et al., 2008).

The use of low molecular weight model nucleophiles in place of peptides addresses some of the above limitations associated with use of peptide reactivity assays. Relative binding of a chemical skin sensitizer is not dependent on the protein/peptide nature of the nucleophile, but rather follows the HSAB (hard and soft (Lewis) acids and bases) concept which allows

for the use of model low molecular weight chemical nucleophiles as protein surrogates to quantify reactivity of electrophilic agents. The HSAB theory and its relevance to several toxicity endpoints have recently been reviewed by Lopachin et al. (2012). The use of relative reactivity does not depend on identification of the target proteins that are covalently modified in the skin allowing for the use of either model peptides or other nucleophiles in the development of *in chemico* assays. Enoch et al. (2008) discusses the importance of using model nucleophiles in a recent review. A high throughput kinetic profiling assay reported by Roberts and Natsch (2009) utilized a model peptide to determine second order rate constants as a quantitative end point. Solubility problems, which are common in these reactivity assays, were addressed in this method. Schwobel et al. (2011) published an extensive review which highlights the importance of incorporating reactivity based assays in the prediction of a chemical's toxicity such as skin sensitization. The review discusses the importance of using model nucleophiles and the influence of experimental factors on the determination of quantitative end points such as rate constants. Extensive reviews on skin sensitization and the development of non-animal based assays based on chemical reactivity, which results in covalent protein binding, have recently been published by Organization for Economic Cooperation and Development (2012a,b). The importance of proper chemical categorization is highly encouraged in these reviews.

The use of 4-nitrobenzenethiol (NBT), which is a “soft” thiol based nucleophile, to quantify reactivity of more than 20 electrophilic skin sensitizers from different mechanistic domains was previously reported from our laboratory (Chipinda et al., 2010) where correlation of reactivity to LLNA potency was demonstrated across all domains. NBT reactivity to Schiff Base Formers and diones was predictably absent as these chemicals are harder electrophiles with preferential reactivity to amine based nucleophiles. This study reports the utility of pyridoxylamine (PDA), a hard nucleophile, to complement NBT for identification of electrophilic skin sensitizers. Reactivity of electrophilic chemicals spanning the S_N1/S_N2, Schiff Base Formers (SBF) and acylating agents (AA) mechanistic domains, among others, is discussed in terms of its correlation to LLNA data compiled by Kern et al. (2010) and Gerberick et al. (2005).

2. Materials and methods

2.1. Chemicals

Phosphate buffer, acetonitrile (ACN), pyridoxylamine dihydrochloride (PDA; CAS # 524-36-7) and all test chemicals which were reagent grade were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA) and used without further purification. With a few exceptions, chemicals with available LLNA data were chosen as the test chemicals for reactivity with PDA.

2.2. UV/vis spectroscopy

Absorbance measurements were carried out on a Beckman DU 800 Spectrophotometer (Beckman Coulter Inc., Somerset, NJ) using quartz cells with calibrated 1 cm path lengths. Experiments were carried out at 25 °C with temperature being controlled by a Fisher Scientific Model 9000 circulating water bath (Thermo Fisher Scientific Inc., Waltham, MA).

Reaction progress was followed by monitoring the loss of the amine PDA at 324 nm, where it has its highest molar absorptivity coefficient (ε). Test chemicals were dissolved in acetonitrile at concentrations ranging from 1 to 10 mM. These solutions (250 μL) were combined with 50 μL of 0.1 mM PDA in phosphate buffer (PB) (pH 7.4) and a further 200 μL of PB in a cuvette. The aqueous content of all the experiments was fixed at 50%. PDA was thus the limiting reagent in the reactions. Control experiments contained test chemical, acetonitrile and phosphate buffer to determine background absorbance before each experiment was initiated. Five replicates were performed for each chemical at each concentration. Fifty seven test chemicals consisting of known skin sensitizers, non-sensitizers and pre/prohaptens were used to evaluate the potential of this kinetic assay for identification of skin sensitizers.

2.3. Fluorescence spectroscopy

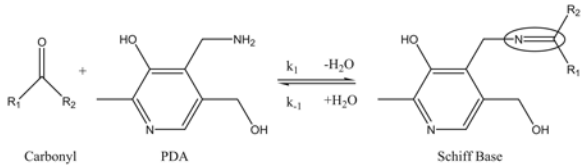
Experiments were performed on a Perkin Elmer Luminescence Spectrometer LS50B (Perkin Elmer Inc., Waltham, MA) with a Czerny quartz lamp. Quartz cuvettes with calibrated 1 cm path lengths were used and a circulating water bath temperature control was set at 25 °C. Excitation of PDA was set at 324 nm with emission at 398 nm. Excitation and emission slit widths were set at 10. Test chemicals were dissolved in acetonitrile at concentrations ranging from 1 to 10 mM. These solutions (250 μL) were combined with 50 μL of 0.1 mM PDA in phosphate buffer (PB) (pH 7.4) and a further 200 μL PB + 1500 μL ACN:PB (50:50) in a 1 cm path length cuvette. Control experiments contained test chemical, acetonitrile and phosphate buffer to determine background fluorescence before each experiment was initiated. Five replicates were performed for each chemical at each concentration. The fluorescence measurements used PDA and test chemical concentrations that were 10-fold less than in the absorbance measurements while maintaining the test chemical:PDA concentration ratios in the reaction mixtures.

2.4. Rate constant determination

The amount of amine remaining at time t, $[\mathrm{PDA}]_t$, was calculated using the following equation (N1);

$$[\mathrm{PDA}]_t = \frac{\boldsymbol{A}_{\mathrm{PDA}_t}}{\varepsilon} \text{ or } [\mathrm{PDA}_t] = \frac{\boldsymbol{F}_{\mathrm{PDA}_t}}{\boldsymbol{k'}} \quad \text{(N1)}$$

where $\boldsymbol{A}_{\mathrm{PDA}_t}$ and $\boldsymbol{F}_{\mathrm{PDA}_t}$ are the absorbance and fluorescence of PDA at time t, ε (7800 ± 64 $\mathrm{M}^{-1}\ \mathrm{cm}^{-1}$) is its absorptivity co-efficient and k' ($70 \times 10^6\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$) is the PDA fluorescence constant which is a product of the proportionality constant (k), the incident light intensity (I_0), the PDA quantum yield ($\boldsymbol{\Phi}$), and absorptivity co-efficient (ε) where the path length is 1 cm. Calculation of kinetic rate constants were based on the assumption that stable adducts are formed between PDA and the electrophiles (E). The nucleophilic attack by the free amine on PDA on electron deficient centers on the E was assumed to be the major reaction pathways as represented by the example of Schiff base formation between a dicarbonyl such as glyoxal (which is the E) with PDA.



The following rationale was used to calculate the apparent pseudo-first order rate constant k_a for PDA – *E* reactions from which the observed rate constant k_{obs} was determined;

$$\mathrm{PDA}+\boldsymbol{E}\underset{\boldsymbol{k}_{-1}}{\overset{\boldsymbol{k}_1}{\rightleftharpoons}}\mathrm{PDA}-\boldsymbol{E} \quad \text{(R1)}$$

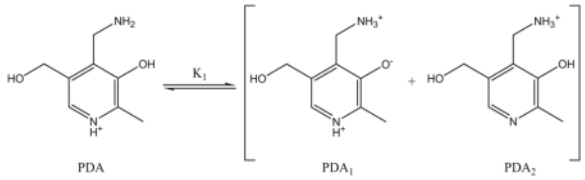
The reaction rate for (R1) can be written as

$$\frac{-\boldsymbol{d}[\mathrm{PDA}]}{\boldsymbol{dt}}=\boldsymbol{k}_1[\mathrm{PDA}][\boldsymbol{E}]-\boldsymbol{k}_{-1}[\mathrm{PDA}-\boldsymbol{E}] \quad \text{(N2)}$$

Assuming the formation of a stable adduct which resulted in the observed loss of PDA absorbance it implies that $k_1 > k_{-1}$ which reduces equation (N2) to (N3).

$$\frac{-\boldsymbol{d}[\mathrm{PDA}]}{\boldsymbol{dt}}=\boldsymbol{k}_1[\mathrm{PDA}][\boldsymbol{E}] \quad \text{(N3)}$$

PDA will establish the equilibrium Eq. (1) in solution with the resultant equilibrium constant K_1 defined by equation (N4) as:



(1)

$$\boldsymbol{K}_1=\frac{[\mathrm{PDA}]}{[\mathrm{PDA}_1]+[\mathrm{PDA}_2]} \quad \text{(N4)}$$

If the mass balance for PDA which is $[PDA]_T = [PDA] + [PDA_1] + [PDA_2]$ is considered and substituted into equation (N4) to rewrite the equation in terms of $[PDA]_T$ and [PDA] the result is equation (N5) which can be used to rewrite the reaction rate equation (N3) in terms of total amine $[PDA]_T$ present to give equation (N6):

$$[\mathrm{PDA}]=\frac{\boldsymbol{K}_1[\mathrm{PDA}]_{\boldsymbol{T}}}{1+\boldsymbol{K}_1} \quad \text{(N5)}$$

$$\frac{-\boldsymbol{d}[\mathrm{PDA}]_{\boldsymbol{T}}}{\boldsymbol{dt}}=\frac{\boldsymbol{k}_1\boldsymbol{K}_1[\mathrm{PDA}]_{\boldsymbol{T}}[\boldsymbol{E}]}{1+\boldsymbol{K}_1} \quad \text{(N6)}$$

When all constants are grouped the apparent rate constant is defined as equation (N7) thus reducing equation (N6) to equation (N8) from which the integrated rate equation (N9) for pseudo-first order reaction between PDA and the test chemical *E* is obtained.

$$\boldsymbol{k}_{\mathrm{a}}=\frac{\boldsymbol{k}_1\boldsymbol{K}_1[\boldsymbol{E}]}{1+\boldsymbol{K}_1} \quad \text{(N7)}$$

$$\frac{-\boldsymbol{d}[\mathrm{PDA}]_{\boldsymbol{T}}}{\boldsymbol{dt}}=\boldsymbol{k}_{\mathrm{a}}[\mathrm{PDA}]_{\boldsymbol{T}} \quad \text{(N8)}$$

$$\ln\left\{\frac{[\mathrm{PDA}_{\boldsymbol{T}}]}{[\mathrm{PDA}]_{\boldsymbol{T}_0}}\right\}=-\boldsymbol{k}_{\mathrm{a}}\boldsymbol{t} \quad \text{(N9)}$$

3. Results

The depletion of PDA was measured by monitoring PDA absorbance at 324 nm after rapid mixing by adding 450 μL of electrophile in ACN:PB to 50 μL of PDA. For chemicals whose absorbance interfered with that of PDA the reactivity was measured using fluorescence where PDA was excited at 324 nm and emitted at 398 nm. Table 1 lists rate constants of the electrophilic chemicals (*E*) from five mechanistic domains which are (i) Schiff Base Formers (SBF), (ii) Michael acceptor (MA), (iii) S_N1/S_N2 reactants, (iv) S_NAr and (v) acylating agents (AA) which were reacted with PDA under pseudo-first order conditions ([PDA] ≪ [*E*]). The average time taken for the PDA – *E* reaction to reach completion ranged from <1 min (BQ) to >2 h (SA) at 1:5 PDA:*E* concentration ratios. The reactions were considered to have reached completion when the PDA absorbance change with time remained constant. Fig. 1(i) and (ii) show loss of PDA during reaction with glutaraldehyde (GDH) and glyoxal (GXL) representing the SBF reactants.

Fig. 2(i) and (ii) gives, as an example, the linear plots of k_a versus [*E*] (equation (N7)) derived from the integrated rate equations for PDA reactions with GDH and GXL from which the slope ($k_{obs} = k_1K_1/(1 + K_1)$) is obtained. Table 1 lists the calculated observed rate constants (k_{obs}) for all the tested chemicals. The same rationale was used for kinetics that were measured using fluorescence. The (k_{obs}) for the fluorescence experiments are highlighted (superscript b) in Table 1. Where linear curve fitting was not satisfactory, quadratic regression was used and the *x* co-efficient was adopted as the slope k_a (equation (N9)) as has been reported by Roberts and Natsch (2009).

3.1. Correlation with LLNA data

The LLNA EC3 values (Anderson et al., 2010; Chipinda et al., 2008; Dearman et al., 2000; Gerberick et al., 2005; Kern et al., 2010; Roberts et al., 2007a,b) for the test chemicals were converted to molar pEC3 values by dividing the molecular mass (*Mw*) for the test chemical by the EC3 value and finding the log (pEC3 = log (*Mw*/EC3)) (Roberts and Natsch, 2009). The pEC3 values for the test chemicals listed in Table 1 were plotted against log (k_{obs}) values to determine the relevance of the rate constants to the chemicals' potency as skin sensitizers. A plot of pEC3 versus log (k_{obs}) values for 25 test chemicals is shown in Fig. 3. A highly significant correlation between potency in the LLNA and reactivity to PDA, independent of chemical mechanistic domain, was observed as indicated by the following linear equation (with statistical parameters);

$$\mathrm{pEC3}=0.39(\pm0.05)\log(\boldsymbol{k}_{\mathrm{obs}})+2.82(\pm0.11);\boldsymbol{n}=25,\boldsymbol{R}^2=0.76,\boldsymbol{R}^2_{\mathrm{adj}}=0.75,\boldsymbol{s}=0.48,\boldsymbol{F}=72.2 \quad \text{(N10)}$$

Regression analysis of pEC3 versus log (k_{obs}) for chemicals within the same mechanistic domains resulted in R^2 values ranging from 0.77 to 0.87. However, comparison of the resultant linear slopes and intercepts (*t*-statistic) had no statistically significant differences in regression lines across the mechanistic domains. There is therefore a clear overall trend in the correlation between reactivity and LLNA potency, regardless of whether the regression analysis is done within the same mechanistic domain or by combining all mechanistic domains. The relatively small difference in individual mechanistic domain R^2 versus inclusive R^2 values, and overlapping data between domains (Fig. 3) suggests that reactivity is an important parameter in the determination of allergenic potency. It must be stated, however, that due to the limited number of chemicals tested per mechanistic domain, a definitive conclusion concerning the value of the ranking of potency within mechanistic domains cannot be made from the present study. What is also apparent is the variation within the data (Fig. 3) where chemicals with almost identical k_{obs} have been reported to have greater than 10-fold differences in the EC3 values. The observed variations may have been due to hydrophobicity (log *P*) values of the test chemicals which were not included in the regression equation (N10) whereas the LLNA derived EC3 values depend on both an allergen's ability to get absorbed through the skin (hydrophobicity) and haptenate skin proteins (reactivity). Some mechanistic domains may therefore require a hydrophobicity parameter to model partitioning between aqueous and lipid compartments of the epidermis; and this was not factored in when the regression equation (N10) was derived. There was no observed PDA reactivity with non-skin sensitizers such as sulfanilamide, benzaldehyde and chlorobenzene among others (Table 2). Lack of reactivity to the moderate and weak skin sensitizers such as ethyl acrylate and tetraethylthiuram disulfide which are in the Michael acceptor and disulfides domains, respectively, was also observed as expected because soft electrophiles such as ethyl acrylate and disulfides preferentially react with thiols over amines. This was, however, in contrast to the reactivity which was observed with hydroxyethyl acrylate which has been shown to be a moderate skin sensitizer in the LLNA. Prohaptens listed in Table 3 did not react with PDA. The lack of PDA reactivity for 2,4-dihydroxychalcone is a false negative result as this test chemical is a Michael acceptor which would be expected to be amine reactive.

The chemicals which reacted with both PDA and NBT, together with PDA and NBT rate constants are shown in Table 4. Both NBT and PDA rate constants resulted in the regression equations for NBT (Chipinda et al., 2010) and PDA (equation (N10)) which were used to calculate the predicted pEC3 values for the chemicals listed in Table 4. Fig. 4 demonstrates that there is also an apparent, statistically significant, positive correlation between PDA and NBT predicted pEC3 ($R^2 = 0.52$; $p = 0.043$). However, an R^2 value of 0.52 resulting from an $N = 7$ may be considered a weak correlation which cannot be used to predict the likelihood of a PDA reactive chemical to be reactive to NBT as well. A definitive occurrence/or lack of correlation could only be demonstrated if an $N \gg 7$ chemicals observed herein had reacted with both PDA and NBT.

4. Discussion

The target moieties for haptenation, on proteins, are usually accessible amines or thiols. In most cases, skin sensitizers that are hard electrophiles (e.g. Schiff Base Formers) preferably bind to amines whereas soft electrophiles will bind to thiols. This PDA reactivity method is similar to the peptide depletion assay[7] in that quantitative measurement of protein haptenation by an electrophilic chemical forms the basis of hazard identification for skin sensitization. The reported results herein complement our previously published results with NBT (Chipinda et al., 2010) allowing for the detection of harder electrophiles which do not react with the softer thiol based NBT nucleophile. PDA reactivity experiments considerably expanded the data set of chemicals that could be tested in the absorbance and fluorescence based reactivity method.

The use of PDA as a nucleophile is not without precedence as it is used as a drug with strong nucleophilic potential to scavenge endogenous electrophiles such as ketoaldehydes, dicarbonyls, aldehydes, ketones and α-oxoaldehydes that are overproduced in biological tissues during diabetes associated degenerative diseases (Amarnath et al., 2004; Voziyan and Hudson, 2005). The inhibitory activity of PDA is due to its strong nucleophilicity which was demonstrated when it competitively scavenged carbonyl compounds sparing protein side chains such as Ace-Phe-Lys (Adrover et al., 2009). PDA, just like NBT, is soluble in both the phosphate buffer (pH 7.4) and acetonitrile that were used as the reaction media making it a suitable nucleophile to measure reaction kinetics in solution. Serial absorbance scans of PDA in either the ACN, PB or 50:50 (ACN:PB) were able to show that it is stable for several days. Effects of water on reactivity may have resulted with test chemicals such as anhydrides that tend to react with water since the reaction environment was 50% aqueous. PDA, compared to the thiol nucleophile, is less susceptible to oxidative side reactions which may contribute to the overall depletion of PDA.

The primary amine on PDA has been demonstrated to react via the same mechanism as that for the amino group in peptides such Ac-Phe-Lys (Adrover et al., 2009). The measured rate constants for PDA were reported to be five-fold greater than Ac-Phe-Lys due to the higher proportion of reactive form of PDA at pH 7.4 (Eq. (1)). The pKa of PDA is 10.7 (Vilanova et al., 2004) and the Henderson–Hasselbalch implies that >99.95% of PDA will exist mainly as the free amine at pH of 7.4. This makes PDA a suitable nucleophile for amine reactive electrophiles. The rate constants (k_1) for the test chemicals could have been calculated using

the slope of the plot of equation (N7), that is, ($k_{obs} = k_1K_1/(1 + K_1)$)$x$ and a known value K_1 for the equilibrium. However, no additional value for the purposes of determining relative reactivity to PDA for all the tested chemicals is derived from k_1. The k_{obs} values are sufficient as a measure of amine reactivity.

The strong correlation ($R^2 = 0.76$) between PDA reactivity and LLNA data for the 25 electrophilic chemicals (out of 35 test chemicals; Table 1) demonstrates the viability of using PDA to screen amine reactive skin sensitizers. Despite having only a limited number of chemicals within each domain the overall trend exhibited in Fig. 3 ($R^2 = 0.76$) highlight the importance of allergen reactivity to skin sensitization potency. However, given the wide range of EC3 values (Anderson et al., 2010; Chipinda et al., 2008; Dearman et al., 2000; Gerberick et al., 2005; Kern et al., 2010; Roberts et al., 2007a,b) within and across mechanistic domains, the use of amine rate constants alone may not be sufficient to accurately model the potency of some electrophilic skin sensitizers in the absence of hydrophobicity parameters.

To a large extent, HSAB theory predicts that use of PDA alone would lead to false negative results with known skin sensitizers that are soft electrophiles, particularly electrophiles which belong to the MA and disulfide mechanistic domains. Reactivity of the seven test chemicals listed in Table 4 to both PDA and NBT seems to contradict the HSAB theory, and shows that some electrophiles are neither amine nor thiol selective even though reactivity to thiols is usually much faster. PDA's greater reactivity compared to protein lysines (Adrover et al., 2009) may be the reason for the observed reactivity of PDA with thiol-reactive compounds that may otherwise not be reactive to lysine. In as much, use of PDA as a nucleophile may potentially result in some false positive results with respect to identification of protein nucleophile binding sites and also under classify the allergenic potency in the absence of testing with NBT; however, it would still correctly identify the chemical as an allergen. It is possible that chemicals that react very slowly (i.e. very weak electophiles) may not cause *in vivo* sensitization that would require establishment of threshold reactivity below which sensitization will not occur. It is also important to note that the seven non-sensitizers tested in this method did not react with PDA. The greater reactivity of PDA compared to lysine, therefore, makes it a suitable probe for deriving a kinetics based model for predicting sensitizers.

Fig. 4 demonstrates a correlation, albeit a weak one, between reactivity to PDA and NBT. The predicted pEC3s shown in Table 4 also suggest that PDA kinetics predict pEC3 better than NBT kinetics and this may be because PDA has greater reactivity when compared to amines such as lysine. Notably, the PDA predicted pEC3 of HEA (=1.97) in Table 4 was closer to the LLNA pEC3 (=1.92) than the NBT predicted pEC3 (=0.83), contradicting the finding reported by Roberts and Natsch (2009) that HEA is well predicted by cysteine peptide kinetics. The contradiction may be attributed to the fact that Roberts and Natsch (2009) developed a Michael acceptor only regression whereas equation (N10) is for mixed mechanistic domains. The argument that PDA kinetics give better predictions than NBT kinetics cannot be conclusively stated in light of the few chemicals ($n = 7$) that were compared. Comparison of the pEC3 PDA and pEC3 NBT with LLNA pEC3 (Table 4) demonstrates that neither NBT nor PDA rate constants alone are sufficient to predict the

LLNA EC3 with better accuracy across all mechanistic domains. These findings underscore the importance of using both the thiol and amine based nucleophiles in this reactivity method.

Chemicals reacted with PDA and NBT must be electrophilic for covalent modification of the nucleophiles to occur. In the current form, the PDA/NBT assays will give false negative results for metals (which react via co-ordinate bonding) and prohaptens (Table 3) which require a bioactivating step to produce electrophilic metabolites prior to the reactions with the nucleophiles. The performance of this assay is therefore restricted to data sets where the chemical species are mainly electrophilic.

There is a need to develop quantitative high-throughput *in chemico, in vitro* and *in silico* approaches which will be utilized as predictive assays to meet required non-animal based chemical hazard identification regulatory requirements. It is important that a reactivity based method be able to quantitatively screen small sets of structurally similar chemicals as well as many chemicals with diverse structures without necessarily rationalizing the underlying sensitization mechanism. In addition, it is desirable that *in chemico* assays be capable of providing potency information from which a reactivity threshold can be linked to the *in vivo* threshold required for the induction of skin sensitization. The positive correlation demonstrated between log (k_{obs}) and pEC3 suggests that the reactivity methods can potentially be utilized for both hazard identification and potency ranking of skin sensitizers. Developing a much larger reactivity data set will allow categorization of test chemicals into their respective mechanistic domains whereby rate constants can be used for potency ranking within the same mechanistic domain.

Read-across (Schultz et al., 2009) and (Q)SARs (Enoch et al., 2008) approaches are regarded as important instruments for generating information on the intrinsic properties of chemicals under the REACH legislation (Combes et al., 2003) but often times there is lack of the required relevant physicochemical and toxicological information to adequately perform these analysis for test chemicals. The ease with which the PDA and NBT reactivity experiments can be performed avails a simple and timely *in chemico* assay that can be used to collect reactivity data which can be incorporated into QSAR models that are used to predict the skin sensitization potency of the test chemicals. Development of both the amine and thiol based nucleophiles to determine reactivity of electrophilic skin sensitizers fits well in the current paradigm on toxicity testing where emphasis for alternative methods is shifting to predicting the skin sensitizing potential (and ranking) of chemicals that lack toxicological and exposure data. The challenge to create ways to efficiently predict skin sensitizing potency of these chemicals requires quantitative information on the reactivity of the chemicals. The advantages of the use of PDA and NBT as nucleophiles to identify electrophilic skin sensitizers over other alternative assays include low assay cost, direct monitoring of skin sensitizer binding without the need to separate reactants and product(s), the ability to quantify reactivity across mechanistic domains and skin sensitizer potency spectrum. The total assay time is also significantly reduced. PDA reactivity data, together with NBT reactivity data, can be incorporated into integrated testing strategies comprising of multiple elements tasked to facilitate skin sensitizer identification while reducing costs and animal testing. Whilst this PDA reactivity method cannot replace the LLNA because of

limitations such as the inability measure prohaptens and metals, reactivity information derived from this *in chemico* assay can also be utilized in a weight-of evidence approach or as a preliminary screening assay (Van Den Heuvel et al., 2012) for electrophilic sensitizers (van Leeuwen and Patlewicz, 2007).

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Abbreviations

PDA	pyridoxylamine
NBT	nitrobenzenethiol
ACD	allergic contact dermatitis
LLNA	local lymph node assay
SBF	Schiff Base Formers
MA	Michael acceptor
S_N1/S_N2	Nucleophilic Substitution (1 or 2)
S_NAr	Nucleophilic Substitution (aromatic)
AA	acylating agents

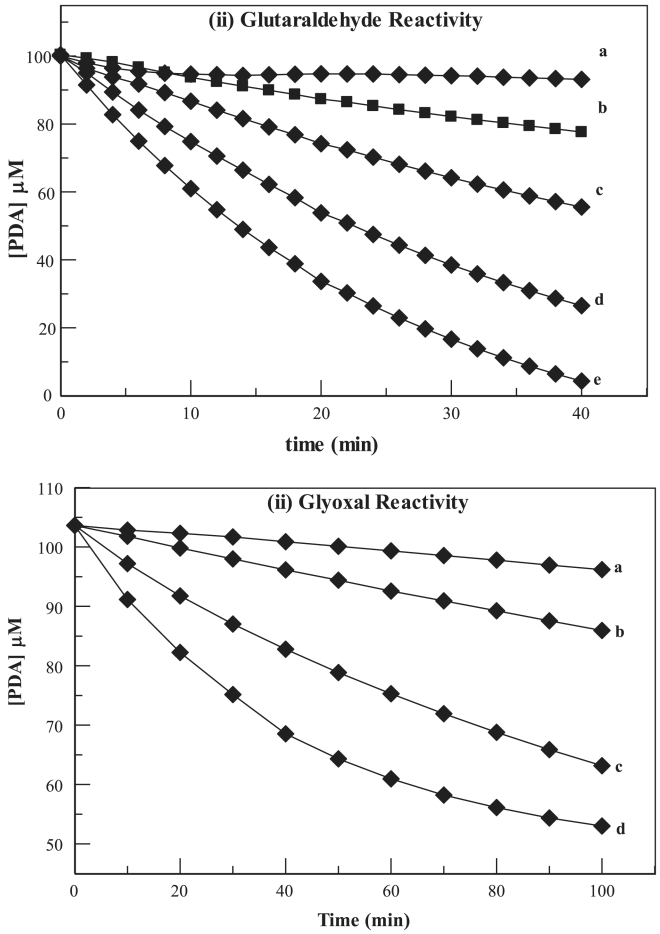

Fig. 1.

Example of PDA reactivity. (i) Glutaraldehyde reactivity to 0.1 mM PDA was observed with (a) 1 mM, (b) 2 mM, (c) 4 mM, (d) 8 Mm and (e) 10 mM glutaraldehyde in 50:50 ACN:PB (pH 7.4). (ii) PDA (0.1 mM) was reacted with (a) 1, (b) 2, (c) 4, and (d) 8 mM glyoxal in 50:50 ACN:PB (pH 7.4).

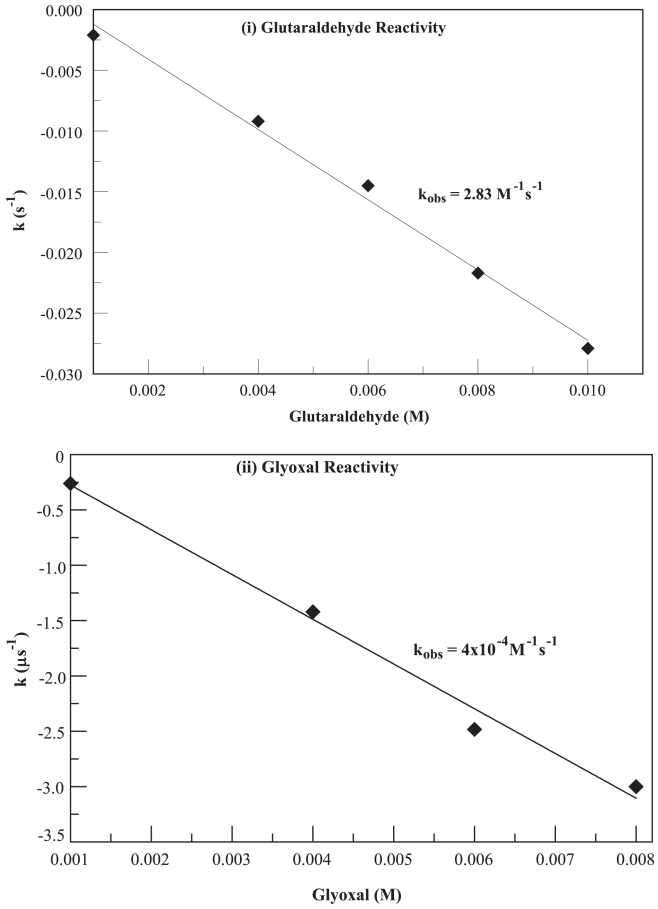

Fig. 2.

Linear plots derived from the integrated rate equation plots for PDA reactions with (i) glutaraldehyde and (ii) glyoxal. The observed rate constants (k_{obs}) calculated from the slopes of the curves were 2.83×10^{-4} M^{-1} s^{-1} and 4.04×10^{-4} M^{-1} s^{-1} for glutaraldehyde and glyoxal, respectively.

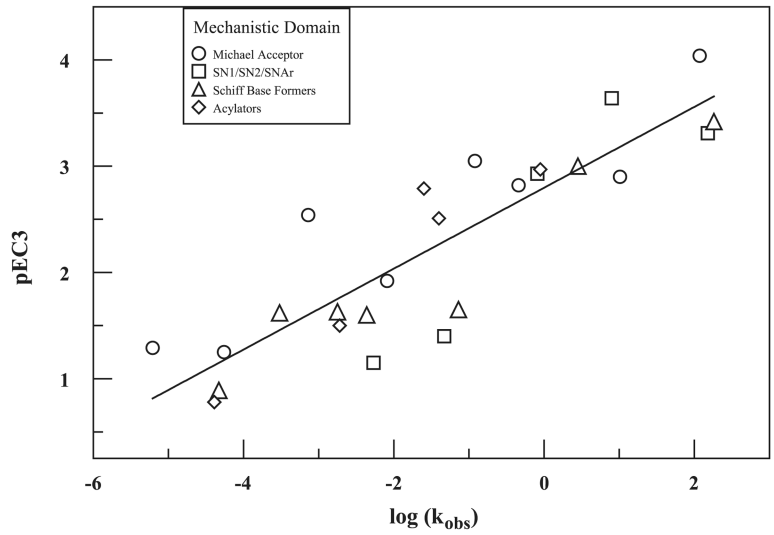

Fig. 3.

Log (k_{obs}) versus pEC3 for the MA, AcA, SBF and S_N1/S_N2 domains listed in Table 1. There was strong correlation ($R^2 = 0.76$) between PDA reactivity and potency in the LLNA. The seven chemicals which did not react with PDA and three chemicals without LLNA data were not included in this plot. pEC3 = log {MW/EC3}.

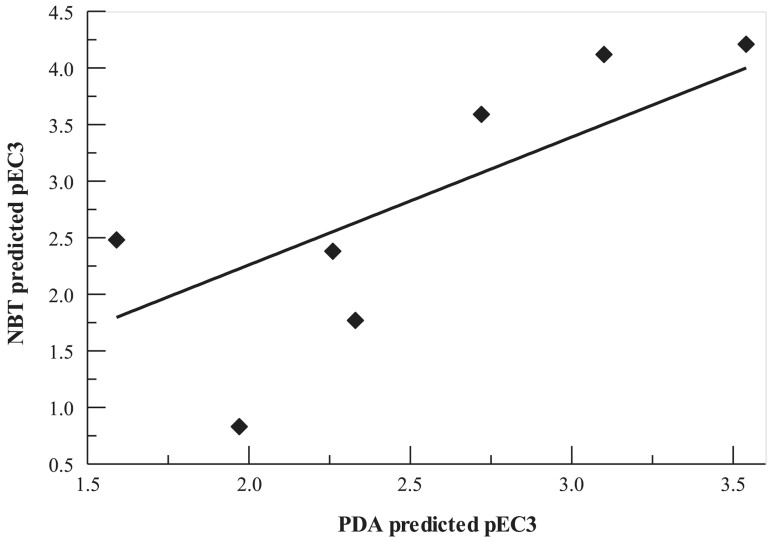

Fig. 4.

PDA versus NBT predicted pEC3 values for chemicals in Table 4. The pEC3 values are calculated using NBT and PDA regression equations (Ref. (Chipinda et al., 2010) and equation (N10)). A positive correlation (R^2 = 0.59, p = 0.043) between the pEC3s was obtained.

Table 1

Test chemicals and rate constants derived from overall reaction rates for PDA.

Michael acceptors	CAS #	MW (g/mol)	EC3 (%)	pEC3[a]	k_{obs} (M^{-1} s^{-1})	log(k_{obs})	LLNA potency class
Benzoquinone (BQ)	106-51-4	108.09	0.0099	4.04	116	2.07	ext
Hydroquinone (HQ)	123-31-9	110.11	0.14	2.90	10	1.01	str
Lauryl Gallate (LG)[b]	1166-52-5	338.44	0.3	3.05	0.12[b]	−0.92[b]	str
Propyl gallate (PG)	121-79-9	212.20	0.32	2.82	0.46	−0.34	str
2,4-Dihydroxychalcone (DHC)	1776-30-3	240.25	0.56	5.05	NR	NR	str
Phenyl cinnamaldehyde (PCA)	1210-39-5	208.26	0.60	2.54	7.3×10^{-4}	−3.14	str
Hydroxyethyl acrylate (HEA)	818-61-1	116.12	1.4	1.92	8.1×10^{-3}	−2.09	mod
Linalool aldehyde (LLA)[b]	Not Known	168.24	9.5	1.25	5.5×10^{-5}[b]	−4.26[b]	weak
Hexylcinnamaldehyde (HCA)[b]	101-86-0	216.32	11	1.29	6.2×10^{-6}[b]	−5.21[b]	weak
Benzyl cinnamate (BCT)	103-41-3	238.29	18	1.11	NR	NR	weak
Ethylacrylate	140-88-5	100.12	28	0.55	NR	NR	weak
Methyl methacrylate	80-62-6	100.12	90	0.05	NR	NR	weak
SN1/SN2/SNAr							
Dinitrofluorobenzene (DNFB)	70-34-8	186.10	0.032	3.76	NR	NR	ext
Nitrobenzyl bromide (NBB)	3958-60-9	216.03	0.05	3.64	7.9	0.90	ext
Cyanuric Chloride (CNC)	108-77-0	184.41	0.09	3.31	150	2.18	ext
Benzyl bromide (BB)	100-39-0	171.03	0.2	2.93	0.81	−0.09	str
Tetraethyldicarbamoyl sulfide (TEDCD)	Not known	264.4	1.7	1.40	4.7×10^{-2}	−1.33	mod
Methyl methane sulfonate (MMS)[b]	66-27-3	110.13	8.1	1.15	5.3×10^{-3}[b]	−2.27[b]	mod
Schiff base formers							
O-pthalaldehyde (OPA)	643-79-8	134.13	0.05	3.42	180	2.26	ext
Glutaraldehyde (GDH)	111-30-8	100.12	0.1	3.00	2.8	0.45	str
Formaldehyde (FDH)	50-00-0	30.03	0.61	1.65	0.072	−1.14	str
Glyoxal trimer (GXL)	4405-13-4	210.14	1.4	1.62	4.0×10^{-4}	−3.52	mod
Methyl pyruvate (MPVT)	600-22-6	102.09	2.4	1.63	1.8×10^{-3}	−2.75	mod
Phenylacetaldehyde (PAC)	122-78-1	120.15	3	1.60	4.4×10^{-3}	−2.36	mod
2,3-Butanedione (BD)	431-03-8	86.09	11	0.89	4.7×10^{-5}	−4.33	weak

Michael acceptors	CAS #	MW (g/mol)	EC3 (%)	pEC3[a]	k_{obs} (M^{-1} s^{-1})	log(k_{obs})	LLNA potency class
Acylators							
Acetic anhydride (AA)	108-24-7	102.09	ND	ND	14	1.15	str
Pthalic anhydride (PA)	85-44-9	148.12	0.16	2.97	0.89	−0.05	str
Maleic anhydride (MA)	108-31-6	98.06	0.16	2.79	25×10^{-3}	−1.6	str
Trimellitic anhydride (TMA)[c]	552-30-7	192.13	0.6	2.51	0.04	−1.40	str
Palmitoyl chloride (PMC)	112-67-4	274.87	8.8	1.50	1.9×10^{-3}	−2.72	mod
Oxalic acid (OA)[b]	144-62-7	90.03	15	0.78	4.1×10^{-3} [b]	−4.39[b]	weak
Succinic anhydride (SA)	108-30-5	100.07	ND	ND	2×10^{-5}	−4.70	ND
Diethylphthalate (DEP)	84-66-2	222.24	ND	ND	6.1×10^{-6}	−5.21	ND
Disulfides							
Tetraethylthiuram disulfide (TETD)	97-77-8	296.54	5.5	1.73	NR	NR	mod
Aminophenyl disulfide (APDS)	1141-88-4	248.37	ND	ND	NR	NR	ND

[a] pEC3 derived from LLNA EC3 values reported in the literature (Anderson et al., 2010; Chipinda et al., 2008; Dearman et al., 2000; Gerberick et al., 2005; Kern et al., 2010; Roberts et al., 2007a,b).

[b] Fluorescence spectrophotometry was used to measure PDA depletion. ND – LLNA EC3 not reported, NR – no reactivity measured, ext – extreme, str – strong, mod – moderate.

[c] TMA is also reactive as a Michael acceptor.

Table 2

Non-sensitizers which did not react with PDA.

Chemical name	CAS #	MW (g/mol)	PDA
Sulfanilamide (SFA)	63-74-1	172.2	NR
Benzaldehyde[a] (BDH)	100-52-7	106.12	NR
Chlorobenzene (CB)	108-90-7	112.56	NR
Sodium lauryl sulphate (SLS)	151-21-3	288.38	NR
Acetonitrile (ACN)	75-05-8	41.05	NR
Glycerol (GCL)	56-81-5	92.09	NR
Acetone (ACT)	67-64-1	58.08	NR
Methyl salicylate (MS)	119-36-8	152.15	NR

[a]Non-sensitiser in the LLNA even though chemical is a reported human sensitizer (Natsch et al., 2012). The depiction (−) means no reactivity to PDA was observed.

Table 3

Purported pre/prohaptens tested for PDA reactivity.

Chemical name	CAS #	MW (g/mol)	PDA	Pre/prohapten
Aniline (ANL)	62-53-3	93.1	NR	+
2-Mercaptobenzothiazole (MBT)	149-30-4	167.24	NR	+
Cinnamic alcohol (CA)	104-54-1	134.18	NR	+
4-Nitrobenzene-1,2-diamine (NBDA)	99-56-9	153.14	NR	+
Imidazolidinylurea (IDDU)	39236-46-9	388.29	NR	+
1,4-Phenylenediamine (PPD)	106-50-3	108.14	NR	+
Eugenol (EU)	97-53-0	164.2	NR	+
Isoeugenol (IEU)	97-54-1	164.2	NR	+
Dihydroeugenol (DHEU)	2785-87-7	166.22	NR	+
Limonene (LNN)	5989-27-5	136.24	NR	+
Thioglycerol (TGCL)	96-27-5	108.16	NR	+
Benzyl salicylate (BSL)	118-58-1	228.24	NR	+
3,4-Dinitrophenol (DNP)	577-71-9	184.11	NR	+
Nickel chloride ($NiCl_2$)	7718-54-9	129.60	NR	(metal)

NR – no reactivity measured. The depiction (+) means the chemical is a pre/prohapten.

Table 4

Chemicals which reacted with both PDA and NBT.

Chemical name	log(k_{obs}) PDA	log(k_{obs}) NBT	pEC3[a] PDA	pEC3[a] NBT	LLNA pEC3
Benzoquinone (BQ)	2.07	3.22	3.54	4.21	4.04
Nitrobenzyl bromide (NBB)	0.90	3.10	3.10	4.12	3.64
Benzyl bromide (BB)	−0.09	2.39	2.72	3.59	2.93
Tetraethyldicarbamoyl sulfide (TEDCD)	−1.33	0.79	2.26	2.38	1.40
Formaldehyde (FDH)	−1.14	−0.03	2.33	1.77	1.65
Hydroxyethyl acrylate (HEA)	−2.09	−1.28	1.97	0.83	1.92
Methyl methane sulfonate (MMS)	−2.82	1.06	1.59	2.48	1.15

[a] pEC3 estimated from NBT (Chipinda et al., 2010) and PDA (equation (N10)) regression equations.